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EFFECTS OF PHORBOL ESTERS AND LIPOPOLYSACCHARIDE ON ENDOTHELIAL CELL MICROFILAMENTS: LASER SCANNING CONFOCAL MICROSCOPY AND QUANTITATIVE MORPHOMETRY OF DOSE DEPENDENT CHANGES

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The disruptive action of phorbol esters on microfilament integrity was used to develop a comparison of dosage effects with effects on the modulation of the phosphoinositide turnover and protein kinase C regulatory system. The novel technique of laser scanning confocal epifluorescence was used to study fiber orientation in phorbol ester treated cells. We treated endothelial cells with control agents and agents known to stimulate protein kinase C: 4α-phorbol, phorbol 12-myristate 13-acetate (PMA), phorbol dibutyrate (PDB), or lipopolysaccharide. After incubation with the test agents, the endothelial cell microfilaments were stained with rhodamine phalloidin and viewed by conventional epifluorescence and by laser scanning confocal epifluorescence microscopy. The images obtained by the confocal microscopy corresponded to a thin optical section through the cells, 300 nm or more in thickness. The microfilaments extended predominantly in the plane of focus. After exposure

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of the cells to phorbol esters, the stress fibers became more parallel in arrangement or were fragmented, but remained in the plane of focus. The modification of microfilaments in response to phorbol esters was quantitated by a single blind analysis. In order to compare the morphological changes with a biochemical action of the phorbol esters, we measured phosphoinositide turnover. The dose-dependence of morphological changes was compared and contrasted to the dose-dependent effect of phorbol esters on bradykinin-stimulated phosphoinositide turnover. PMA had about the same EC_{50} (1-5 nM) for both biochemical and morphological processes. -PDB was less potent in inducing the disruption of microfilament structure than in inhibiting phosphoinositide turnover. Lipopolysaccharide was ineffective in inducing a morphological change under these conditions. A simple activation of protein inase C is insufficient to explain the dose-dependent effects of phorbol esters. Thus a morphometric analysis can help distinguish the potency of modulators.

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TABLE OF CONTENTS

INTRODUCTION

Morphometry has importance in careful comparisons of effects of chemically related agents. We selected to use the effect on microfilaments mediated by calcium-diacylglycerol-dependent protein kinase (protein kinase C) (Schliwa et al., 1984; Fey and Penman, 1984; Kellie et al., 1985) to evaluate various agents. The disappearance of stress fibers in monkey kidney BSC-1 cells treated with phorbol 12-myristate 13-acetate (PMA), [also named 12-0-tetradecanoylphorbol-13-acetate], but not in cells treated with the biologically inactive 4α-phorbol, was observed by Schliwa et al. (1984) using immunofluorescence and high voltage electron microscopy. The morphological changes were independent of changes in microtubules, intermediate filaments or fibronectin and were not influenced by changes in other second messengers such as cyclic AMP or calcium levels.

The phorbol ester class of tumor promoters mimic the endogenous intracellular messenger diacylglycerol by stimulating protein kinase C (Berridge, 1984; Nishizuka, 1986). Lipopolysaccharide also activates protein kinase C (Wrightman and Raetz, 1984). Cells derived from the vascular endothelium respond to bradykinin and other hormones by an increase in phosphoinositide metabolism (Lambert et al., 1986; Derian and Moskowitz, 1986; Nielsen and Wood, 1988). In this pathway, phosphatidyl inositol-4,5 bisphosphate is hydrolyzed to form two second messengers, inositol 1,4,5-triphosphate and diacylglycerol, the intracellular activator of protein kinase C. Bradykinin stimulation of phosphoinositide turnover may lead to release of prostacyclin and smooth muscle relaxing factor (Vanhoutte et al., 1986).

In order to evaluate phorbol ester action, we compared changes in the cellular cytoskeleton induced by phorbol esters using conventional epifluorescence and the new method of laser scanning confocal epifluorescence microscopy (White et al., 1987). In laser scanning confocal epifluorescence microscopy, argon ion laser light passes through a virtual pinhole and scans

in a raster pattern over the sample. The fluorescence emitted by the specimen is integrated over time into a complete image. An advantage of the confocal system is that out-of-focal plane fluorescent structures do not contribute to the image. This method may result in an ability to optically section through the sample with higher resolution in the x, y and z dimensions (White et al., 1987).

MATERIALS AND METHODS

Cell culture and staining. A bovine aortic endothelial cell line, GM 7372, originally cloned and transformed by Grinspan et al., (1983) was obtained from the NIGMS Human Genetic Mutant Cell Repository at the Institute for Medical Research, Camden, NJ, USA and routinely cultured in Medium 199 containing 20% fetal bovine serum. Cells were prepared for incubation and staining by modification of a previous method (Nielsen et al., 1985). Briefly, cells were trypsinized and seeded onto glass cover slips 1-4 days prior to incubation in the absence or presence of phorbol esters (Pharmacia P-L Biochemicals), bradykinin (Calbiochem, La Jolla CA) or lipopolysaccharide (Sigma, St. Louis MO). After suitable incubation, the cells on cover slips were fixed in 3.7% formaldehyde (TEM grade, Tousimis, Rockville, MD) in Dulbecco's Phosphate buffered saline (D-PBS) for 10 min at room temperature. The cells on cover slips were then washed briefly in D-PBS, extracted with cold acetone (4 min), air dried and incubated with rhodamine phalloidin (3 μM in D-PBS, Molecular Probes, Eugene, OR). The cover slips were mounted with glycerol: D-PBS (9:1), pH 8.5 and viewed by epifluorescence.

Morphologic evaluation. The dose response of agents was investigated single-blind as follows. After staining, the samples were randomly labeled with a code. The morphology was then evaluated using conventional epifluorescence by another individual unaware of the code. The cover slips were scanned from bottom to top, alternately left to right and right to left, until a total of

>200 cells per cover slip were scored. Only cells completely within the field of view were evaluated. The cover slips were decoded and then the data averaged and plotted as a function of the agent concentration.

Phosphoinositide metabolism. Phosphoinositide turnover was measured as described by Nielsen and Wood (1988). Briefly, confluent endothelial cells at 37°C were loaded with [^{3}H]-myo-inositol in Krebs-Ringer bicarbonate for 4 hr. LiCl (10 mM final, Berridge et al., 1982) and phorbol esters were then added as appropriate. The cells were incubated for 15 min with LiCl before bradykinin (100 μM final) was added. Incubation with bradykinin was carried out for 60 min and at the end of that time the medium was removed. Then the cell layer was gently washed with D-PBS containing 10 mM LiCl and the cell layer extracted with cold methanol-HCl (200:1). [^{3}H]-inositol phosphate was isolated by chromatography (Roth et al., 1986). As the phorbol esters were dissolved in dimethyl sulfoxide, this solvent was included in control assays at the same 1% final concentration, but had no effect on the activity. The action of each agent was assessed in three experiments of triplicate wells of cells for each condition.

Epifluorescence and Confocal Epifluorescence Photomicroscopy.

Conventional fluorescence micrographs were taken with a Nikon Optiphot equipped with 40x Fluor objective (n.a. 0.85) and UFX exposure system on Kodak Tri-X film (Eastman Kodak, Rochester, NY) that was developed with ACU-1 developer (Acufine, Chicago IL) containing Kodak anti-fog tablets. Confocal fluorescence micrographs were taken with a Bio-Rad Lasersharp MRC-500 (Bio-Rad, Richmond, CA) using a Zeiss Axiophot microscope equipped with a 63x planAPOCHROMAT, n.a. 1.4. An argon ion laser provided the spot source of excitation light and synchronized moving mirrors generated the sweep pattern. Thirty to fifty sweeps of each image were accumulated and averaged. The images were photographed from a 2000 line flat-screen monitor (MRC 530) and

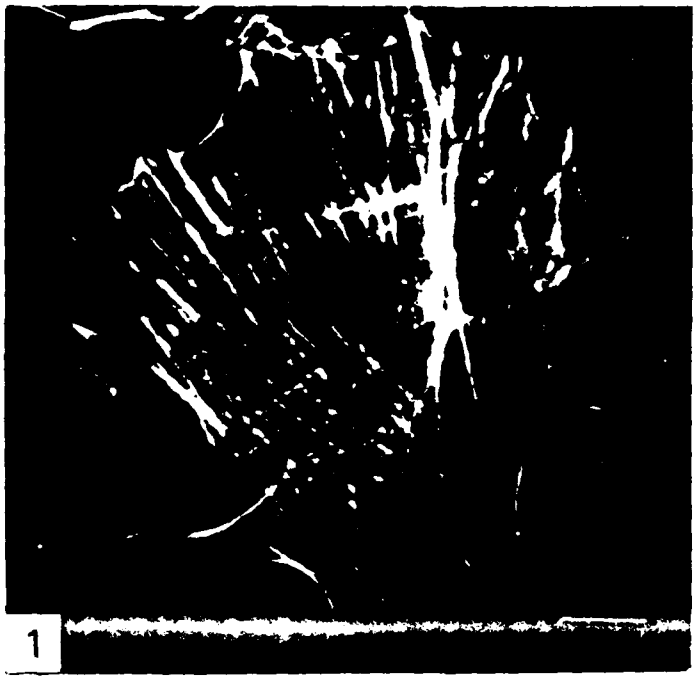
photo phed with Kodak Plus-X film. The Plus-X was developed with Microdol-X. Negatives were printed on Ilfospeed papers (Ilford, Basildon, UK).

Data analysis. Data are expressed as means ± SEM. The significance of differences between agents at matched concentrations were evaluated by the Student's t statistic.

RESULTS

Study of microfilament structure in GM 7372 endothelial cells treated without or with phorbol esters suggested that several patterns or phenotypes were routinely recognizable: By conventional fluorescence microscopy some cells had microfilaments as in Figure 1. These patterns were characteristic of control cells. The filaments stain brightly and are typically oriented in criss-crossing groups. This 'normal' pattern is denoted as Type I. Some cells had more elongated bodies, denoted Type II, figure 2 (solid arrows). Also shown is an adjacent Type V cell (open arrow), with primarily punctate staining. Another cell form was characteristically dominated by a strong parallel arrangement of microfilaments, denoted Type III, figure 3. Other cells contained the microfilaments which appeared shorter than usual, as in Figure 4 a,b, denoted Type IV. The strong orientation of fibers is often missing (note especially the cell denoted by arrows in figure 4b). Some cells were rounded up and typically showed a punctate arrangement of microfilament staining, denoted Type V, as in Figure 5 and as noted in figure 2. A small proportion (typically less than 2%) of cells did not fit readily into any distinct category.

The cells were analyzed by laser scanning confocal epifluorescence microscopy to evaluate details of the changes in fiber orientation. An advantage of the confocal system is that the image focal plane is shallower than the non-confocal system, thereby permitting a better evaluation of spatial location within the cell. Figure 6a illustrates the fluorescence



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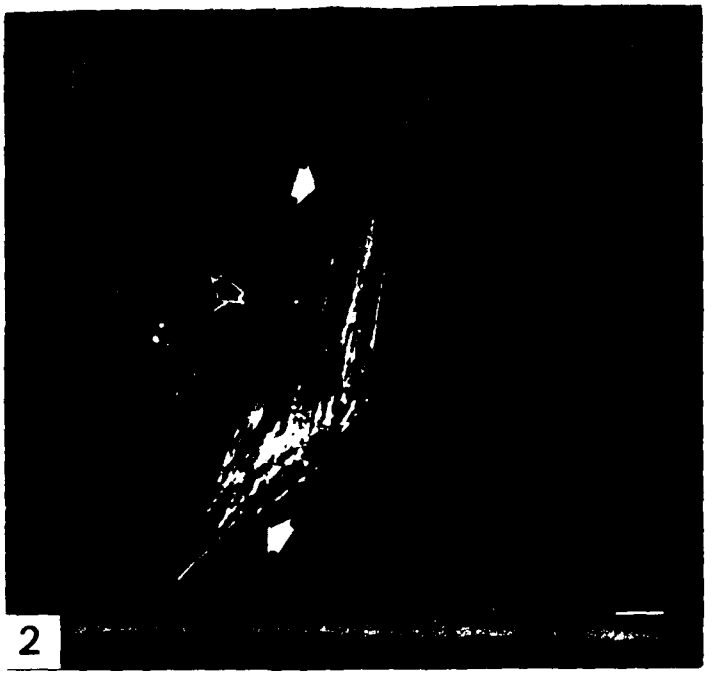

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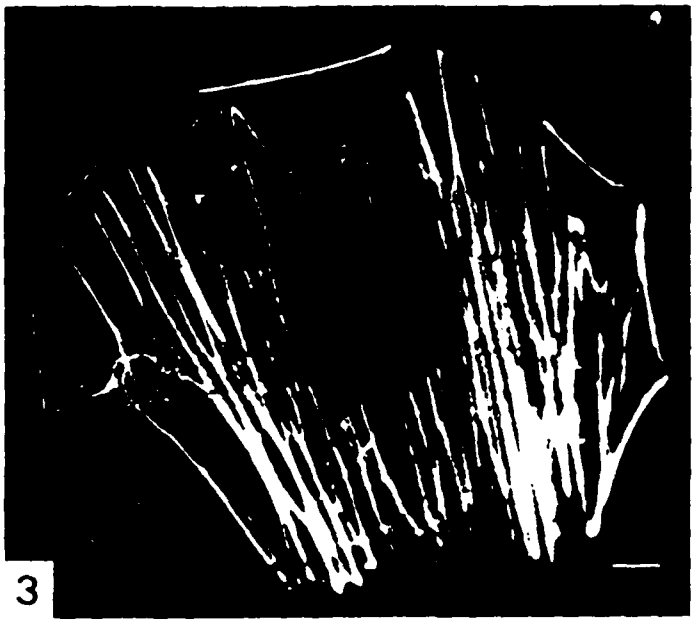

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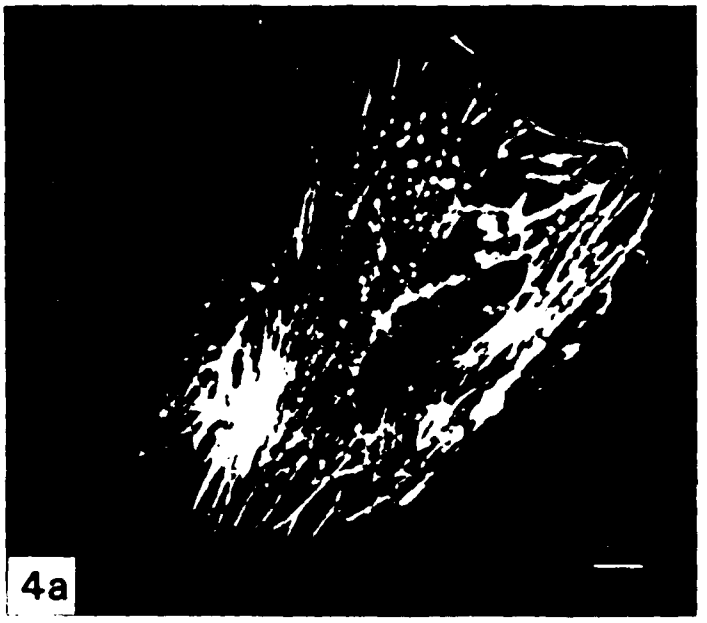

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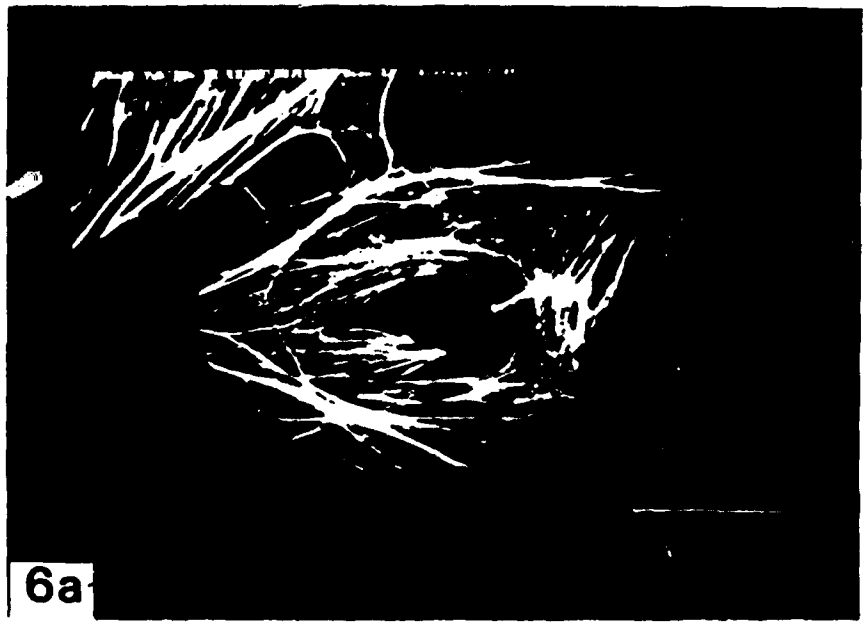

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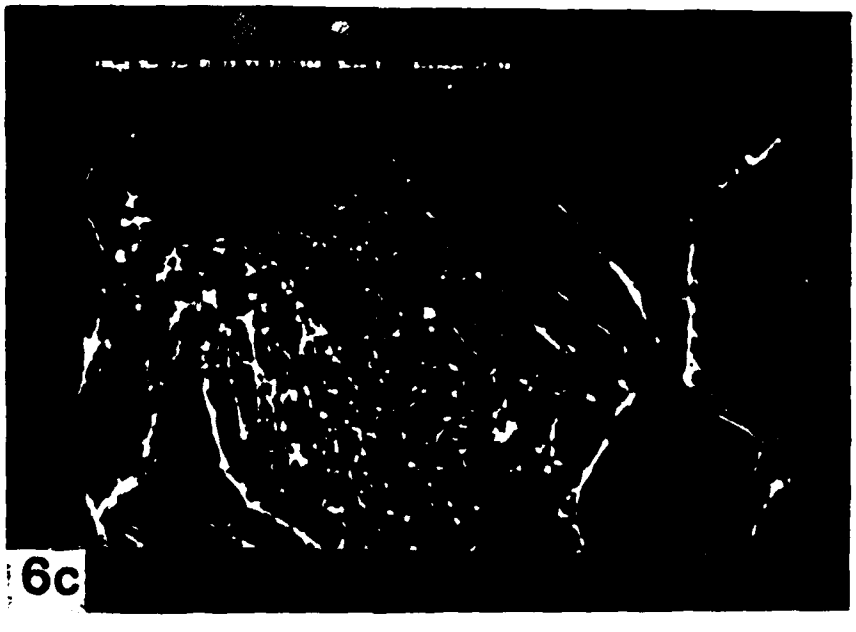
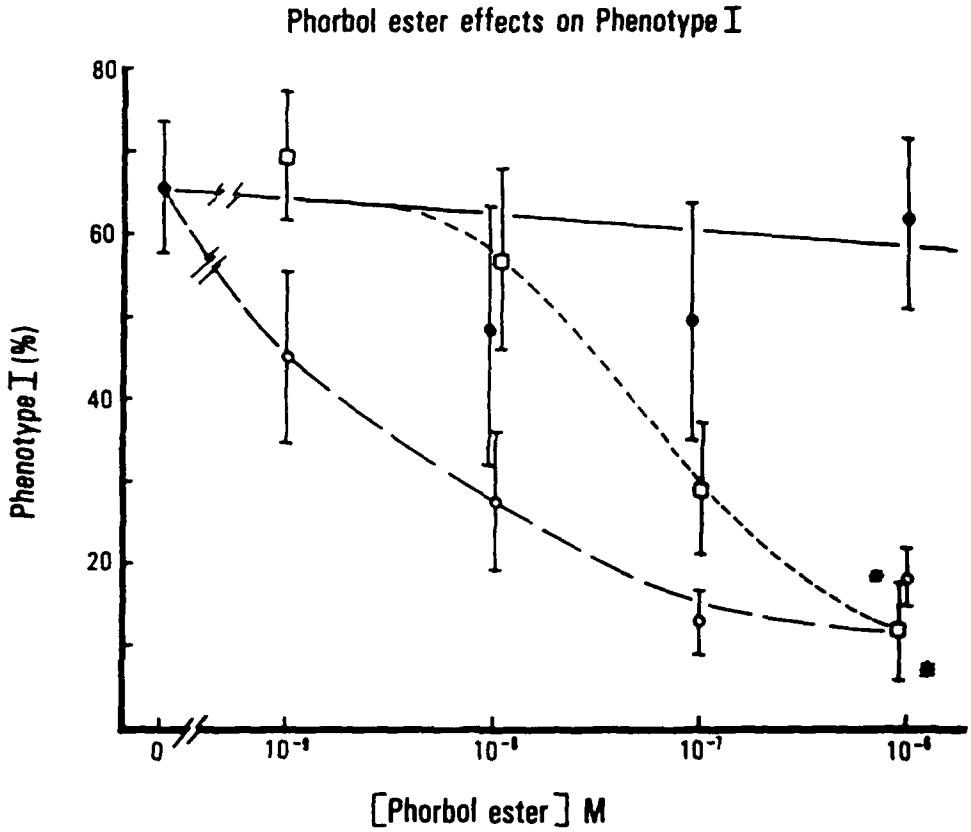


6c

images of microfilaments in a control cell, phenotype I, viewed by laser scanning confocal microscopy. Optical sections adjacent to the one shown contained few fibers (not shown). In addition, comparison of figure 6a with figure 1 also reveals that the fibers lie substantially in the plane of the optical section. Although this is not surprising for flat cells like these, it is important for analysis of changes in the fibers. Figures 6b and 6c illustrate details of the morphology of cells treated with 1μM PMA for 4 hrs. Both phenotype III (Figure 6b) and phenotype IV (Figure 6c) are shown. In both cases the filaments lie substantially in the plane of the optical section, and appear not to have re-oriented into the z-direction.

Control populations of cells consisted of about 70% Type I cell phenotype, almost 20% Type II and 10% all other types combined. PMA decreased the proportion of Type I cells present. The effect was more pronounced at higher doses (Figure 7). The concentration required for a 50% effect (EC_{50}) was about 5 nM for PMA by this test. Phorbol 12,13-dibutyrate (PDB) also reduced the proportion of Type I cells in a dose-dependent fashion (Figure 7), but the agent was less potent than PMA, with an EC_{50} of about 80 nM. The agent 4α-phorbol, which is not a tumor-promoter, had no effect on the proportion of Type I cells present, even at 1μM.

The loss of Type I cells in response to phorbol ester corresponded to an increase in cell Types III and IV. The phenotype characterized by a predominance of parallel stress fibers (Type III) was enhanced by treatment with PMA (Figure 8) at concentrations of 10 nM and above, compared to the control 4α-phorbol. PDB enhanced the Type III phenotype at 1μM. From Figure 9 it is clear that PMA was quite potent in inducing formation of the disrupted microfilament phenotype (Type IV), with a maximal effect at 10 nM. The EC_{50} for this effect was between 1 and 10 nM. The maximum proportion of cells which adopted this phenotype was about 45%. PDB was less potent in producing the Type IV than PMA, but equally effective. The control agent 4α-phorbol was



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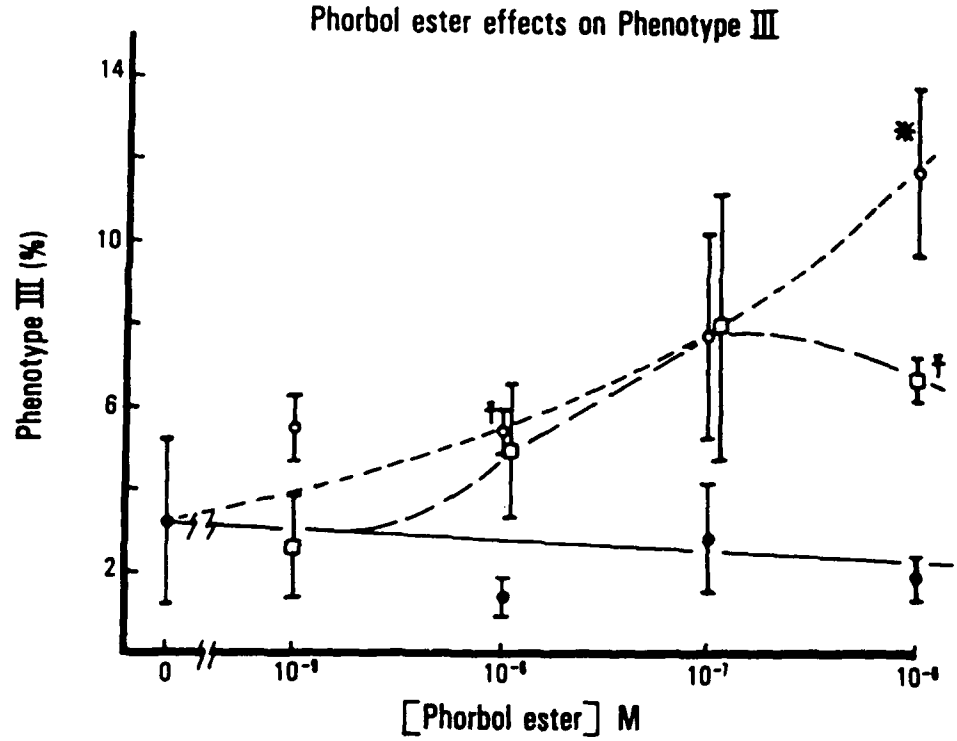
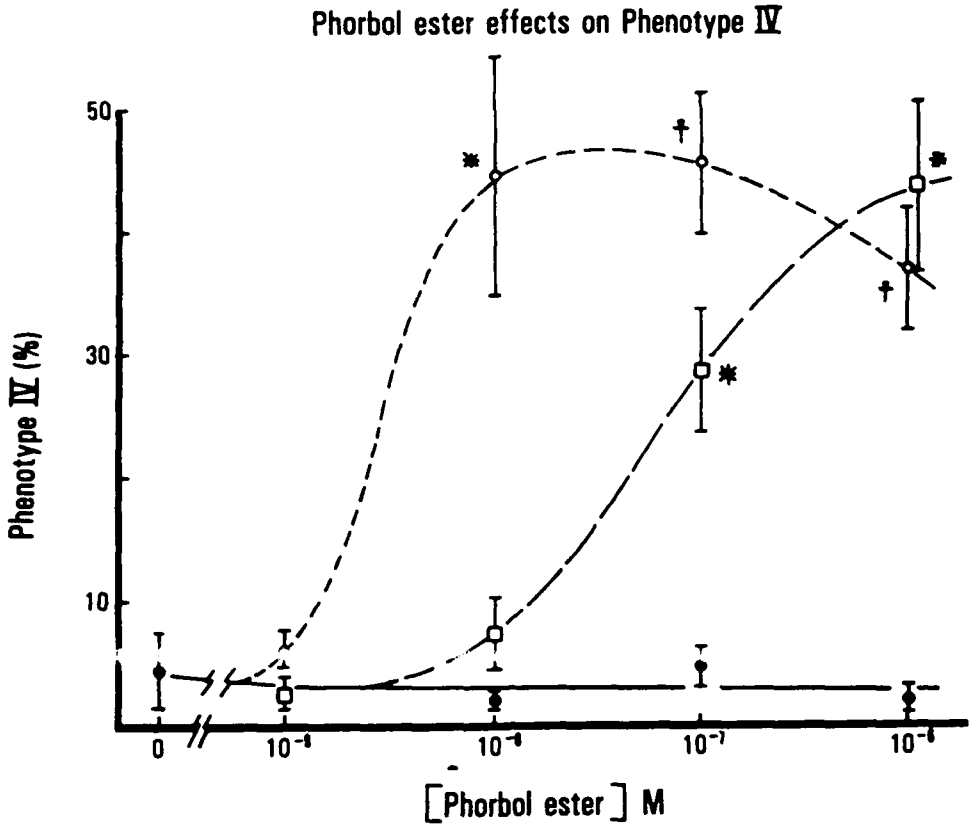
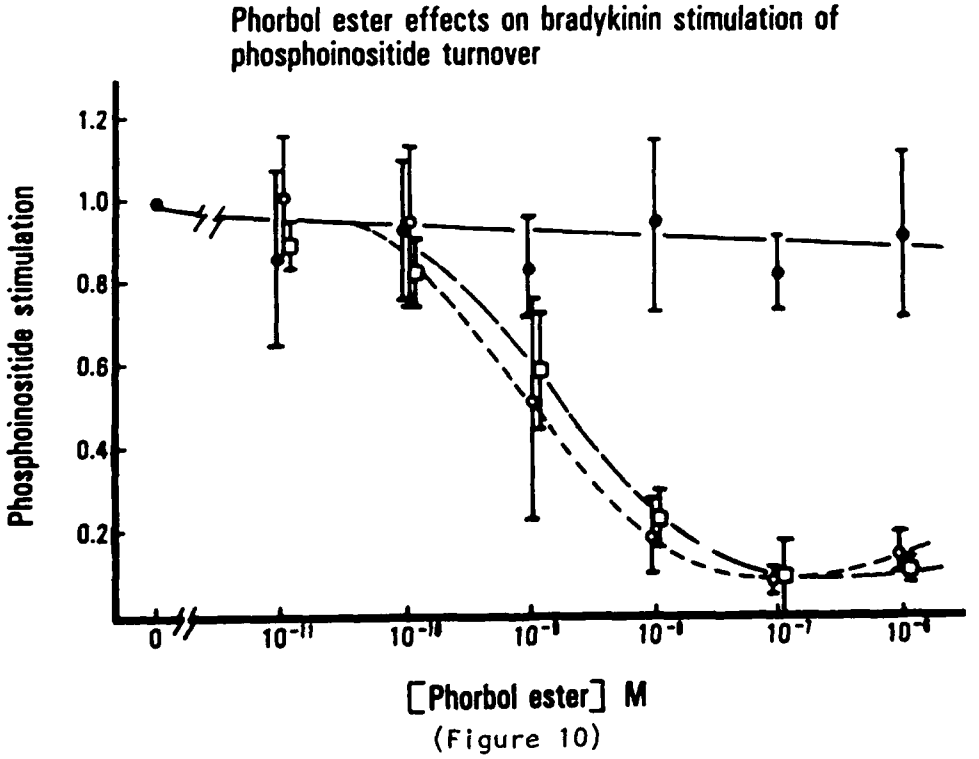


(Figure 8)



(Figure 9)



(Figure 10)

ineffective. The effect of PDB at 10 and 100 nM was not significant. The phorbol esters had no effect on Types II or V (not shown).

We compared the dose responses obtained from these morphometric analyses with a biochemical effect of phorbol esters on these cells in order to correlate biochemical and morphological events. Bradykinin stimulates phosphoinositide turnover in these cells, and both PMA and PDB inhibited the bradykinin-stimulated phosphoinositide inositol metabolism (Figure 10) in a dose-dependent fashion. The EC_{50} for PMA was 1 nM and the EC_{50} for PDB was 2 nM. The agent 4α-phorbol was ineffective at all concentrations up to 1uM. Bradykinin did not affect microfilament morphology. Thus PMA and PDB had almost identical actions on phosphoinositide turnover, yet different, dose-dependent effects on microfilament morphology.

In order to evaluate this morphometric method for another agent that may affect protein kinase C, we treated the cells with lipopolysaccharide. There were no changes in phenotypes II, III, IV or V (not shown). The results with lipopolysaccharide and with PDB suggest that activation of protein kinase C is not sufficient for modification of the microfilament patterns.

DISCUSSION

The laser scanning confocal microscopy produced clearer images of the microfilament structure in both control cells and cells responding to the tumor promoters. Several factors may contribute to this (White et al., 1987). A major factor appears to be the brightness of the fluorescence. Although a video image was produced in one sweep (about 1 sec) of the specimen, accumulation of many sweeps resulted in a noticeable increase in the signal-to-noise ratio. In addition, the difference in the numerical aperture of the objectives used may have made a contribution. Also, the lack of fluorescence contribution from out of focal plane fibers reduced background fluorescence. The thickness of the optical plane in confocal microscopy is variously described as about 700 nm (Brackenhoff et al., 1985) or as thin as 300 nm

(Hook and Odeyale, 1988). The similarity of the microfilaments in the whole fluorescent cells (by conventional epifluorescence) and the single optical slice (by confocal epifluorescence) support the validity of conventional epifluorescence for morphometric analyses of the type shown here. In addition, the confocal microscopy demonstrated that the shortening of the fibers observed in phenotype IV does not result from a re-orientation of the fibers into a more z-like direction (out of the plane of focus). Also the parallel fibers of phenotype III, like many of the cells of type I cells are found in a single optical plane.

Morphometric analyses of microfilament response to phorbol esters were carried out in two different ways: by judging the dose-dependent decrease in the normal population of cells and the dose-dependent increase in two other phenotypes. Both methods showed that phorbol esters altered stress fibers in these cells. The two effects observed were that the length of fibers decreased and that the number of intersections of fibers decreased. Other studies have focused on clarification of the morphological changes and elucidation of factors involved in the changes rather than morphometric analyses (Schliwa et al., 1984; Kellie et al., 1985).

The availability of quantitative morphometric data allows a discussion of mechanistic consequences. The effect of tumor promoters on the bradykinin-stimulated phosphoinositide turnover permits comparison of biochemical and morphological effects. The dose dependent effects of PMA on inositol turnover and morphometric depletion of phenotype I were indistinguishable. In addition, 4α-phorbol was inactive in inducing cytoskeletal changes and inhibiting phosphoinositide turnover. One explanation for these results is that the dissolution of microfilaments in response to PMA may have the same mechanistic basis as the blockade of the phosphoinositol turnover, perhaps the activation of protein kinase C. The dose-dependent appearance of phenotype IV in response to PMA would be consistent with this. In contradiction, PDB would

appear to be quite potent in its effect on stimulated phosphoinositide turnover, but not as potent in its depletion of phenotype I or in its elicitation of phenotype IV. In addition, lipopolysaccharide, also a stimulator of protein kinase C, and bradykinin which stimulates the whole phosphoinositide pathway, did not have the same morphologic consequences as PMA or PDB. It may be that PMA and PDB have differential effects on a process not related to the phosphatidyl inositol-protein kinase C pathway. Such an explanation appears likely for the case of bradykinin, because the inositol 1,4,5-triphosphate produced in response to the peptide releases gelsolin from actin and may result in microfilament stabilization or elongation (Janmey et al., 1987).

A useful aspect of the morphometric analysis derives from a consideration of the sequence of events. Comparison of the total numbers of cells affected makes it clear that the phenotypes III and IV arose from Phenotype I. About 70% of control cells were of phenotype I. At high concentrations of PMA or PDB only 15-20% of the cells retained this phenotype. At high concentrations of PMA or PDB, 45% of the cells were of phenotype IV and 6-12% of phenotype III. Therefore essentially all of the cells which lost type I phenotype adopted types III or IV. One may speculate that phenotype III is an intermediate in formation of phenotype IV. Neither phenotype appears to be a terminal phenotype since prolonged treatment results in detachment of the cells (Gartner et al., 1988), which may be a sequel to phenotype V. Such a sequence would be consistent with our observations and the reported effect of phorbol esters on vinculin (Schliwa et al., 1984).

An objective, machine-based morphometric analysis might be applied to the model system described here, something along the following lines. The definition of fiber length must allow for the gentle curvature often observed in microfilaments. The fiber length must also deal with branch points as

decision points for continuation or termination of the fibers. Absence of intersections may be a partial definition of the parallelism of the fibers. Methods described here may offer a useful method of optimizing pattern recognition and reducing operator bias until more sophisticated machine-based algorithms may be applied. Laser scanning confocal microscopy would provide the clearest images for analysis.

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FIGURE LEGENDS

Figures 1-5. Endothelial (7372) cells stained with rhodamine-phalloidin as described in Experimental Procedures and viewed by conventional epifluorescence microscopy. The bars represent 20 microns. Figure 1, Phenotype I. Figure 2, Two endothelial cells are illustrated, one of Phenotype II (solid arrow) and one of Phenotype V (open arrow). Figure 3, Phenotype III. Figure 4a and b, Phenotype IV. Figure 5, Phenotype V.

Figure 6a,b and c. Laser scanning confocal epifluorescence of endothelial cells stained with rhodamine-phalloidin: a, a cell incubated with 4α-phorbol (1μM). b and c: details of cells incubated with 1μM PMA for 3 hr. The bars represent 20 microns.

Figure 7. Proportion of cells displaying type I morphology after incubation for 3 hr with indicated concentrations of 4α-phorbol (o), PDB ( ) or PMA (o). The data are the results of cell counts on four cover slips at each condition. For the significance tests, *indicates $p < 0.01$, + indicate $p < 0.001$.

Figure 8. Type III morphology. Symbols and experimental details as in Figure 7.

Figure 9. Type IV morphology. Symbols and experimental details as in Figure 7.

Figure 10. Phosphoinositide turnover in endothelial cells stimulated with 10^{-4} M bradykinin: effect of 4α-phorbol (o), PMA (o) and PDB ( ).